ESTERASES, PHOSPHATASES, AND GLYCOGEN IN THE ANTENNAL GLAND OF PACIFASTACUS LENIUSCULUS STIMPSON

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The excretory organs of vertebrates and certain invertebrates possess structural similarities which relate directly to their function. Extensive comparisons exist for the antennal gland of the crayfish on morphological, and to a lesser extent histochemical levels. The ultrastructural studies of Kümmel (1964), demonstrating cells in the coelomosac of the antennal gland similar to podocytes found in the vertebrate glomerulus, support the general contention that this segment of the antennal gland is the site of filtration of the primary urine (Riegel, 1963, 1965; Riegel and Kirschner, 1960). The presence of well-defined brush borders and numerous infoldings of the basal cell membranes in the labyrinth of the antennal gland (Anderson and Beams, 1956) compares to the morphology of the vertebrate convoluted tubule. The localization of alkaline phosphatase both in the brush border of the vertebrate proximal convoluted tubule (Gomori, 1941) and in the brush border of the labyrinth (Kugler and Birkner, 1948; Malaczynska-Suchcitz and Ucinska, 1962) carries the homology to the histochemical level.

This investigation was undertaken to establish the localization of the non-specific esterases, phosphatases and the distribution of glycogen in the antennal gland of the crayfish *Pacifastacus leniusculus* Stimpson. The appearance of a sexual dimorphism involving the esterases prompted the inclusion of disc electrophoresis as a means of validation and characterization.

Materials and Methods

Crayfish collected throughout the year from ponds and rivers near Corvallis, Oregon, were maintained in tanks of running cold water in the laboratory. The temperature in these tanks ranged from 10° C. in the winter to 20° C. in the summer. The size of the gastrolith and appearance of the exoskeleton were recorded to approximate the stage of molt for each animal. A total of 107 animals were used, 51 for histochemistry and 56 for gel electrophoresis.

1. Histochemistry

The antennal glands were fixed either in calcium-formol or in formalin-sucrose (10%–30%) for 18 hours at 4° C. The tissues were rinsed and, due to the very friable nature of the glands, were infiltrated with 15% gelatin at 37° C. for 1½ to 2 hours. Ten-micron sections cut in the cryostat at −20° C. were mounted on

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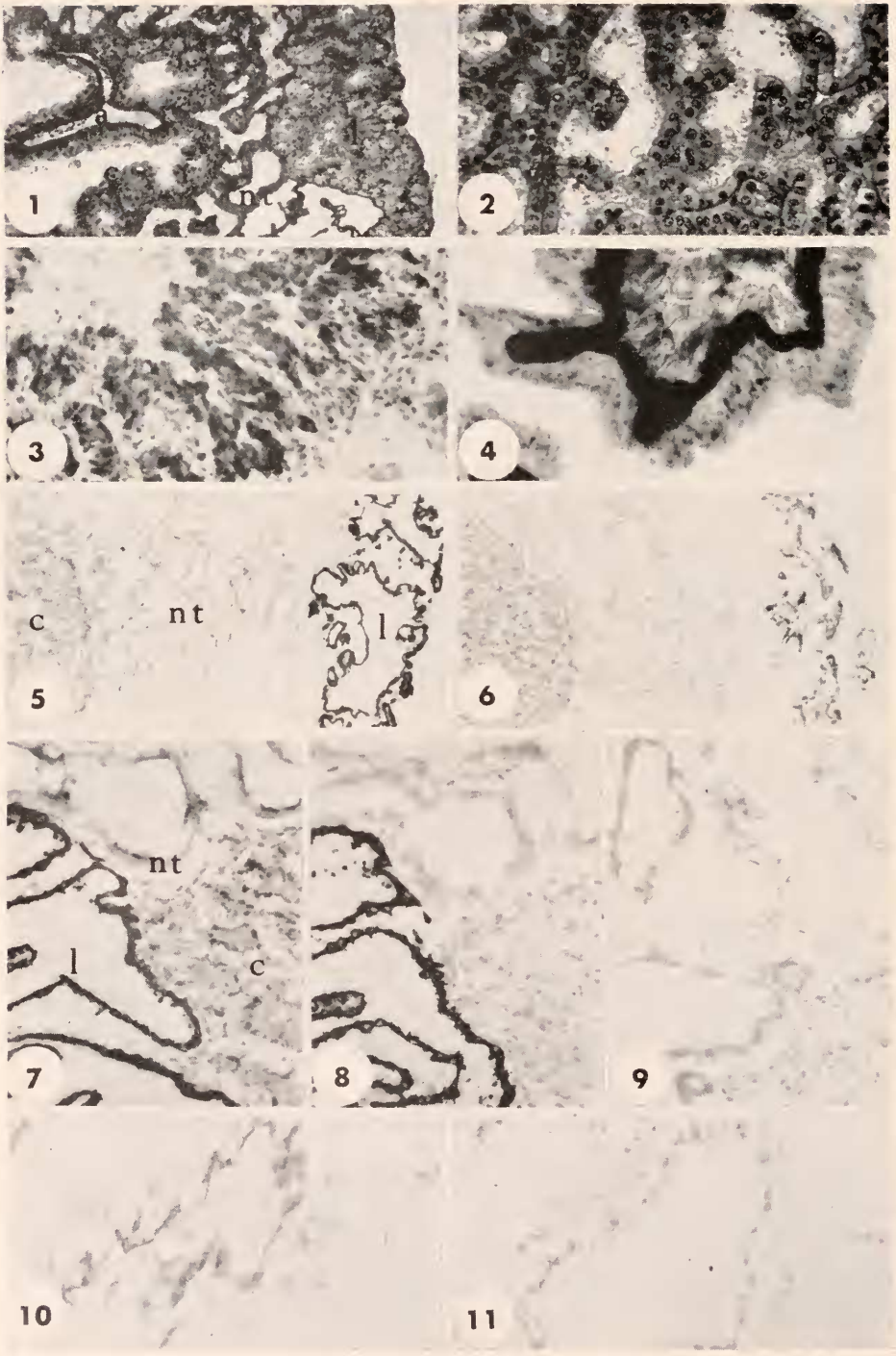

Plate I.

cold slides and air-dried for one to two hours prior to staining. Control slides omitting substrates were processed to evaluate any non-specific staining. Alkaline phosphatase was generally demonstrated by the Gomori calcium-cobalt method but some calcium-formol-fixed tissue was also stained by the azo dye method, using fast violet B as coupler and alpha-naphthyl acid phosphate as substrate. Acid phosphatase was localized by the azo dye method, using alpha-naphthyl acid phosphate as substrate and hexazonium pararosanilin as coupler, and by the Gomori method as outlined by Barka and Anderson (1963).

The non-specific esterases were localized by the alpha-naphthyl acetate method, using hexazonium pararosanilin as coupler (Barka and Anderson, 1963). The inhibitor E 600 (diethyl-p-nitrophenylphosphate) at 10^{-5} *M* in 0.1 *M* tris maleate buffer, pH 7.2, was used to distinguish arom-esterases from the ali-esterases. Eserine sulfate at a concentration of 10^{-5} *M* both in distilled water and in 0.1 *M* phosphate buffer, pH 7.4, was used as an inhibitor of the cholinesterases (Pearse, 1961). Comparable sections were placed in either distilled water, eserine or E 600 for one hour prior to staining. Eserine was either incorporated into or omitted from the incubation medium.

Glycogen was localized by means of the periodic acid Schiff (P.A.S.) procedure as outlined by Pearse (1961). Tissues from six crayfish representing different stages of molt were fixed in Gendre fluid and embedded in paraffin. Comparable sections were subjected to a one-hour digestion in 1% diastase in distilled water prior to the P.A.S. test, to distinguish glycogen from all other P.A.S.-positive diastase-fast material.

2. Electrophoresis

Weighed antennal glands were homogenized in three parts of cold 0.7% saline or in distilled water and centrifuged at 12,000 *g* for 20 minutes. Normally a 100-μl. aliquot of supernatant was subjected to electrophoresis. Glands were also homogenized in 4 or 10 parts of distilled water and 100-μl., 50-μl., 25-μl., and 10-μl. aliquots of supernatant were used with no apparent differences in the final results.

FIGURE 1. Hematoxylin and eosin stained paraffin section of the antennal gland. Coelomosac (c), branch of the reno-antennal artery (a), nephron tubule (nt), and labyrinth (l). × 35.

FIGURE 2. Higher magnification of the labyrinth, demonstrating the apical vesicles both free and attached to cells. × 100.

FIGURE 3. Acid phosphatase reaction in the coelomosac stained by azo dye; formol-sucrose fixation. × 100.

FIGURE 4. Acid phosphatase reaction in the labyrinth. Stained by azo dye method; formol-sucrose fixation. × 430.

FIGURE 5. Non-specific esterase in the antennal gland of the male. Coelomosac (c), nephron tubule (nt), labyrinth (l). Formol-sucrose fixation. × 35.

FIGURE 6. Non-specific esterases in the antennal gland of the female. Formol-sucrose fixation. × 35.

FIGURE 7. Non-specific esterases in the antennal gland of the male. Coelomosac (c), nephron tubule (nt), and labyrinth (l). Formol-sucrose fixation. × 100.

FIGURE 8. Comparable section to Figure 7 treated with 10^{-5} *M* eserine.

FIGURE 9. Comparable section to Figure 7 treated with 10^{-5} *M* E 600.

FIGURE 10. Bladder of crayfish stained for non-specific esterases. Formol-sucrose fixation. × 100.

FIGURE 11. Comparable section treated with 10^{-5} *M* eserine.

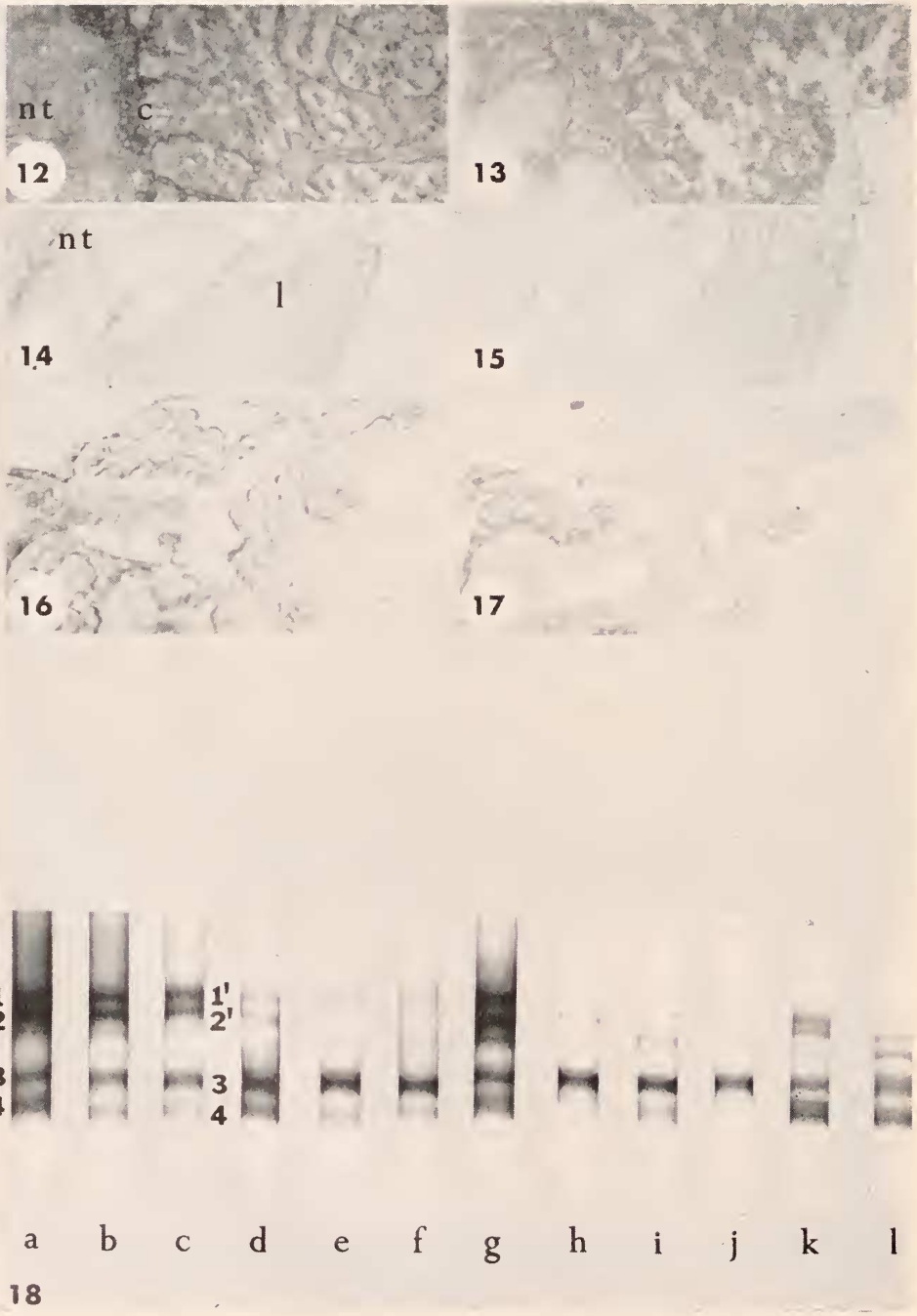

Plate II.

In the case of extremely small antennal glands the entire supernatant constituted one sample.

The apparatus and gels were prepared according to the original recommendations of Davis (1964). The sample gel was replaced by 0.1 ml. of 40% sucrose in tris-glycine buffer containing the supernatant. This mixture formed a layer immediately above the stacking gel and was carefully overlayered with a lighter tris-glycine buffer; an initial current of 0.5 ma. was increased to 1.5 ma. after one hour, at which time the sample had migrated into the large pore stacking gel. The total time for electrophoresis was about three hours at 6° C.

Characterization of the esterases was accomplished by a one-hour exposure to cold 10^{-5} *M* or 10^{-4} *M* eserine sulfate in 0.1 *M* phosphate buffer at pH 7.4 or to 10^{-5} *M* E 600 in 0.1 *M* tris-buffer, pH 7.2, prior to the development of the gels. Control gels were soaked in cold distilled water for the same length of time. Development was accomplished in an incubation medium of the same composition used to demonstrate the esterases histochemically. Since the staining time was 30 to 45 minutes at room temperature, eserine of appropriate concentration was always included in the incubation medium of eserine-inhibited samples to prevent the reversal of the inhibitor during the prolonged incubation. Following a distilled water rinse the gels were photographed by trans-illumination to record the developed electrophoretic patterns, some of which tended to fade in storage.

Results

The reader is referred to Figures 1 and 2 for an orientation to the histology of the antennal gland. More detailed descriptions of the histology appear in papers by Marchal (1892), Peters (1935), Maluf (1939, 1941), and Malaczynska-Suchcitz and Ucinska (1962).

Histochemistry

No obvious differences were observed in the histochemistry which could be correlated with the molt cycle.

1. Phosphatases

Acid phosphatase occurs as abundant granules in the cells lining the lumen of the coelomosac (Fig. 3). In the labyrinth it occupies the luminal border as an

Figure 12. Section stained by P.A.S. procedure. Gendre fixation. Coelomosac (c), nephron tubule (nt). × 100.

Figure 13. Comparable section pretreated with diastase to remove glycogen prior to staining. × 100.

Figure 14. Section of labyrinth (l) and nephron tubule (nt) stained by P.A.S. Gendre fixation. × 100.

Figure 15. Section comparable to the above treated with diastase. × 100.

Figure 16. Bladder stained with P.A.S. Gendre fixation. × 100.

Figure 17. Bladder pretreated with diastase.

Figure 18. Electrophoretic patterns separated by disc electrophoresis. (a) Untreated control, adult male, (b) 10^{-5} *M* eserine, (c) 10^{-4} *M* eserine, (d) untreated control, adult female, (e) 10^{-5} *M* eserine, (f) 10^{-4} *M* eserine, (g) untreated control, adult male, (h) 10^{-5} *M* E 600, (i) untreated control, adult female, (j) 10^{-5} *M* E 600, (k) juvenile male, 45 mm. total length, (l) juvenile female, 45 mm. total length.

intense band and also appears as granules at the base of the cell around the nucleus (Fig. 4). The number and size of the granules vary to some extent between different animals. Some granules were occasionally recorded in the nephron tubule and in the bladder.

Alkaline phosphatase was present only in the labyrinth where it was typically confined to the luminal border. The nuclei presented the false positive reaction characteristic of the Gomori procedure. They were unstained when the azo dye procedure was used.

2. Esterases

Esterases sensitive to both eserine and E 600 are abundant in the cells lining the lumen of the coelomosac. In the labyrinth the non-specific esterases display a sexual dimorphism. The reaction in the male is very intense as compared to a much weaker reaction in the female (Figs. 5 and 6). The stain is diffuse and cytoplasmic with the apical vesicles staining intensely. Treatment with 10^{-5} *M* eserine does not diminish the reaction in either male or female animals but it is abolished completely by 10^{-5} *M* E 600 (Figs. 7, 8 and 9). The bladder and nephron tubule give a slight positive reaction which is completely eserine-sensitive (Figs. 10 and 11).

3. Glycogen

Glycogen is present in all areas of the antennal gland and lumen. However, cells in the periphery of the coelomosac exhibit a particularly intense diastase-labile, P.A.S.-positive stain (Figs. 12 and 13). The bladder epithelium also shows a marked staining for glycogen in comparison to the labyrinth and nephron tubule (Figs. 14, 15, 16 and 17). Additional P.A.S.-positive, diastase-fast materials are present in the nephron tubule, basement membranes, brush borders, and granules in the blood cells.

Electrophoresis

A comparison of the patterns of esterases separated from homogenates of male and female green glands supports the histochemical observations of a sexual dimorphism. In the mature male four bands are observed. The bands 1 and 2 are superimposed on a diffuse background reaction which along with band 4 is somewhat inhibited by exposure to eserine (Fig. 18 a, b, c). E 600 inhibits most of bands 1 and 2 whereas all of band 3 is not affected (Fig. 18 g, h). In the female a significant difference is observed in the bands comparable to bands 1 and 2 in the male. These bands labeled 1′ and 2′ are not as intense and do not respond to the inhibitors in the same manner as those of the male. Band 1′ is insensitive to eserine and E 600, as is its counterpart in the male, and band 2′ is sensitive to eserine (Fig. 18 d, e, f, i, j).

Crayfish of both sexes, judged to be juveniles according to the size criteria of Mason (1963), were examined to establish whether the dimorphism is present prior to their reproductive period. Homogenates of these antennal glands showed electrophoretic patterns very similar to those observed in adult animals (Fig. 18 k, l).

Discussion

The distribution of phosphatases in the antennal gland of the crayfish reported in this study confirms the presence of alkaline phosphatase at the luminal border of the labyrinth and acid phosphatase in the coelomosac as reported by Malaczynska-Suchcitz and Ucinska (1962). However, acid phosphatase was also observed as granules in the cells of the labyrinth as well as at their luminal boundaries.

The presence of both phosphatases in the same cellular area in the labyrinth raises the question as to the validity of assigning integrity to each enzyme or to placing emphasis on the possibility of a single phosphatase capable of responding to both acid and alkaline pH's. McWhinnie and Kirchenberg (1966) reported phosphatase activity in the crayfish hepatopancreas which reflected two peaks of activity: one at pH 6.8 to 7.3 and another at pH 8.0 to 8.5. In the mammalian kidney acid phosphatase and alkaline phosphatase in the proximal convoluted tubule demonstrate brush border staining which was attributed to an "alkaline" phosphatase active at an acid pH (Wachstein, Meisel and Ortiz, 1962).

Acid phosphatase-staining granules of the convoluted tubules of the rat have been accepted as lysosomes on the basis of histochemical and biochemical data (deDuve, 1963). However, the identity of the granular component in the labyrinth and coelomosac of crayfish must remain an open question until equivalent biochemical studies are performed.

The complementation of the topographical distribution of the esterases with an electrophoretic analysis of the molecular species of enzymes present is desirable and informative. However, it must be noted that the two procedures do not necessarily demonstrate the same entity. Histochemical techniques localize the insoluble enzyme fractions whereas electrophoresis demonstrates the soluble fractions (Markert and Hunter, 1959). Crayfish of both sexes exhibited E 600-resistant esterases (arom-esterases) in the electrophoretic patterns. These enzymes may represent formalin-sensitive esterases since they were not observed in sectioned material. Such an E 600-resistant, formalin-sensitive esterase has been reported in mammals (Holt, 1963).

Electrophoretic analysis revealed an E 600-sensitive esterase (ali-esterase) in the kidney of the male mouse not normally present in the females or immature males. Injections of testosterone induced its appearance in these animals (Shaw and Koen, 1963). A sexual dimorphism was also observed in the labyrinth of the crayfish which involved ali-esterases detectable by both histochemical and electrophoretic procedures.

The function of the esterases responsible for the dimorphism in mice or crayfish is unknown. For that matter, the function of the non-specific esterases in general is highly speculative. They may be either hydrolytic or synthetic and since non-specific esterases are usually isolated with the microsomes in cell fractions it has been proposed that they function in protein synthesis (Markert and Hunter, 1959; Hunter *et al.*, 1964).

Riegel (1966) proposed that the antennal gland served both in the excretion and digestion of large molecules. The "formed-bodies" observed in the urine presumably represent the structures in which these processes occur. The labyrinth exhibits secretory activity in the form of blebs or vesicles which separate from the

apex of the cells to lie free in the lumen. A correlation may exist between these activities and the distribution of ali-esterases in the labyrinth of the male. Possibly the esterases associated with the dimorphism are involved in the metabolism and excretion of a substance prevalent in the physiology of male crayfish. That they are specifically localized in the labyrinth suggests a functional differentiation of this segment.

Glycogen was present in all areas of the gland, staining most intensively in the epithelium of the coelomosac and bladder and less intensely in that of the labyrinth and nephron tubule. In addition, it appeared within the lumen, thereby raising questions regarding the mechanisms of its secretion and reabsorption by the antennal gland. Possibly part of the glycogen present in the proximal areas of the gland is released into the lumen as a component of the formed bodies of the coelomosac. Since Riegel's work indicates that the formed bodies are not present in the urine of the bladder, their contents are released for reabsorption or disposal. The greater intensity of the P.A.S. reaction in the bladder epithelium may have relation to the reabsorption of glycogen.

Summary

1. The esterases, acid and alkaline phosphatases, and glycogen were investigated in the antennal gland of the crayfish by histochemical procedures. Gel electrophoresis was employed to further characterize the esterases.

2. A sexual dimorphism occurred in the labyrinth characterized by an intense reaction for the ali-esterases in the male contrasted to a weak reaction in the female. Eserine-sensitive esterases were also observed in the coelomosac, nephron tubule, and bladder. Both alkaline phosphatase and acid phosphatase were localized in the luminal border of the labyrinth. Acid phosphatase was also observed in the coelomosac and as granules in cells of the labyrinth. Glycogen was most concentrated in the coelomosac and bladder but was observed in the other areas of the gland and its lumen in less concentration.

3. The significance of the above reactions was discussed in relation to their roles in the function of the antennal gland.

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